IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re PATENT APPLICATION of
Meyerhoff, et al.

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Examiner: Gupta, Anish

#12
MGJ
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FOR: A PHARMACEUTICAL COMPOSITION CONTAINING PGLU-GLU-PRO-$NH_2$ AND METHOD FOR TREATING DISEASES AND INJURIES TO THE BRAIN, SPINAL CORD AND RETINA USING SAME

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**Declaration Under 37 CFR§ 1.132**

Asst. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

Michael L. Koenig, Ph.D, declares as follows:

1. I am a joint inventor of the subject matter of the above-identified application.

2. I am a biologist at Walter Reed Army Institute of Research in Silver Spring, Maryland and have a Ph.D.

3. I conducted the following experimentation: To show that EEP reduces the damage associated with prolonged excitatory activity, the inventors and I conducted a series of in vitro experiments using prolonged exposure to Glu to excite neuron-rich cultures.

   Materials and methods:

   Materials: TRH, EEP, and Glu were purchased from Sigma, St. Ouis, MO. Reagents used in cell culture and neurotoxicity assays were obtained from Sigma and from Biofluids, Inc., Rockville, MD and GIBCO/Life Technologies, Grand Island, NY.

Primary neuronal cultures: The forebrains and spinal cords of fetal rat pups (embryonic day 15) were isolated and the cells dispersed by repeated trituration in neuronal culture medium (NCM = Ham's F-12: Basal Medium Eagle, 1:1; supplemented with dextrose, 0.6 g/liter; glutamine, 0.35%: and Pen-Strep, 1%). Following centrifugation (900 x g; 5 min.), the cells were plated onto poly L-lysine coated 48 well plates or collagen coated cover glass chamber slides (for imaging experiments) at a density of $10^6$ cells/ml (500,00/$cm^2$). To suppress glial growth, cultures were treated with cytosine arabinoside ($10^{-5}$M) after 3 days in culture. The antimitotic remained on the cultures for at least 4 days, and all cultures were maintained in an incubator (5% $CO_2$; 37°C) for 7 to 10 days prior to use.

Neurotoxicity assays: Neurotoxicity was effected by exposing the cultured neurons to Glu (100μM) in $Mg^{2+}$ -free Locke's solution (NaCl, 154 mM; KCl, 5.6 mM: $NaHCO_3$, 3.6 mM; $CaCl_2$, 2.3 mM; glucose, 5.6 mM; HEPES, 5 mM; pH 7.4) for 30 min (spinal cord neurons) or 60 min (forebrains). The exposure times were chosen to elicit an approximately 50% reduction in viability in the treated neurons relative to culture-matched controls. Following the Glu exposure period, the bathing medium was removed from each well and replaced with fresh Miminum Essential Medium (MEM). Plates were then returned to the incubator overnight. Twenty four h after the initial exposure to Glu ± TRH or Glu ± EEP, the colorimetric dye 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT; Sigma M-2128) was added to each well such that the final concentration of the indicator was 0.15 mg/ml. The reduction of MTT by succinate dehydrogenase (SDH) provides a quantitative measure of cellular viability. SDH in viable cells converts yellow MTT into an insoluble purple formazan product. Plates were returned to the incubator for 1 h at which time unincorporated MTT was removed, and the plates were allowed to air dry. The purple formazan product was then dissolved by adding 250 μl acidified isopropanol (95% isopropanol; 5% 2N HCl), and 200μl aliquots were collected. The absorbance of these aliquots was measured in an ELISA plate reader at λ = 540 nm. Control (untreated) wells were included in every experiment, and viability was calculated as the percentage of mean control values for each plate:

$$\% \text{ Control} = (\text{Test } A_{540}/\text{Mean Control } A_{540}) \text{ X } 100\%$$

Neuroprotection was calculated based on the ability of the test compound to reduce the neurotoxicity attributed to Glu:

$$\% \text{ Neuroprotection} = ((\text{Test } A_{540} - \text{Mean Glu } A_{540})/ (\text{Mean Control } A_{540} - \text{Mean Glu } A_{540})) \text{ X } 100\%$$

Intraneuronal free $Ca^{2+}$ concentrations were determined using the fluorescent $Ca^{2+}$-sensitive dye indo-1. Neurons on collagen-coated cover glass chamber slides were loaded with the membrane-permeable acetoxymethyl ester form of the dye by exposure to a reduced $Ca^{2+}$ (0.2 mM) medium containing indo-1-AM (2μM) for 1 h (37°C), then washed and allowed to recover for at least 15 min. in $Mg^{2+}$-free Locke's solution prior to use.

During all experiments, cells were maintained at a physiological temperature of 37°C. Fluorescence changes in single neurons were monitored using the ACAS 570C confocal laser cytometer (Meridian Instr., Okemos, MI). Indo-1 was excited by laser light in the UV range (351.1-363.8 nm), and emission was detected simultaneously at two wavelengths: 485 nm (free dye) and 405 nm (dye complexed with $Ca^{2+}$). By computing the ratio of emission at detector 2/emission at detector 1, we were able to quantify $[Ca]_i$ in individual neurons without concern for variability due to experimental differences in loading efficiency, cell geometry, or compartmentalization of the dye.

Sequential image scans of fields containing 5-25 neurons (150 X 150 $\mu m^2$) were used to construct kinetic profiles of the effects of Glu ± TRH/EEP on $[Ca]_i$ in primary neuronal cultures. Typically, scans were made at 30 s intervals. To verify adequacy of dye loading and neuronal viability, the $Ca^{2+}$ ionophore ionomycin (2μM) was added 1-2 min before the end of each experiment. Neurons not responding or responding only weakly to the treatment with ionomycin were not included in these studies.

$[Ca]_i$ in these experiments has been expressed in nanomolar (nM) concentrations. Conversion of the ratio values to concentrations was accomplished by preparation of standard curves relating emission ratios to specific extracellular $Ca^{2+}$ standards. Basal $[Ca]_i$ in the neurons was typically just under 100 nM (81 ± 5 nM). "Peak" ratios were defined as the values corresponding to highest levels attained following exposure to Glu. "Post-peak" ratios were measured 5 min after the addition of Glu to provide some indication as to whether $[Ca]_i$ was returning to basal levels or remaining elevated.

**Results:**

Glu-induced neurotoxicity: Excessive amounts of Glu are toxic to neurons in culture. The excitatory amino acid neurotransmitter opens membrane channels providing for a massive influx of both $Na^+$ and $Ca^{2+}$ ions. The resulting depolarization, in turn, opens voltage-gated cation channels, and exhausts neuronal energy reserves, as an effort is made to restore homeostasis. Fig. 1 illustrates the time course of Glu-induced neurotoxicity in vitro. As

early as 30 min following exposure to 100μM Glu, toxicity was apparent, and the number of viable neurons decreased in proportion to the length of time they were exposed to Glu. Likewise, the excitatory amino acid exhibited a dose-dependent toxicity. Fig. 2 reflects the fact that neuronal viability decreased with increasing concentration of Glu with an $LD_{50}$ of approximately 100μM.

Neuroprotection by EEP: As indicated in Fig. 3, neuroprotection against 100μM Glu was readily apparent at EEP concentrations of 500 and 100μM. Although at EEP concentrations of 50 and 100 μM, no protection was evident, 500 μM EEP, given simultaneously with Glu, was protective in neurons derived from both forebrain and spinal cord. The tripeptide was only slightly more protective in neurons derived from forebrain (24.5 ± 7.2%) than in those prepared from spinal cord (20.0± 2.0%). At a two-fold higher concentration of 1000 μM, EEP was substantially more neuroprotective in neurons from forebrain (54.1 ± 5.3%) than in those originating from fetal spinal cord (38.9 ± 4.9%). The differences however were not significant (P = 0.0557).

To compare the neuroprotective efficacy of EEP with that of TRH, experiments were conducted using identical cultures prepared on the same day and maintained in culture for the same amount of time. Neurons from forebrain or spinal cord were treated with Glu (100μM) alone or simultaneously with the excitatory amino acid and either EEP or TRH. The results are presented in Fig. 4. EEP was significantly more neuroprotective than TRH in neurons from both the forebrain (Fig. 4a) and spinal cord (Fig. 4b). AT an EEP concentration of 500μM, MTT-based measurements of neuronal viability were 24.5% greater than that seen following exposure to Glu alone, whereas, at the same concentration, TRH affords only 5.1% protection. The difference is even more apparent at 1000μM. Neuroprotection by EEP at this concentration (54.1%) is almost four times higher than for TRH (14.8%). In neurons derived from spinal cord, 500μM EEP effects 20.0% neuroprotection, whereas the same concentration of TRH is effectively nonprotective; at 1000 μM, EEP (38.9%) is almost eight times more neuroprotective than TRH (5.2%).

Reduction of Glu-stimulated increases in $[Ca]_i$ by TRH and EEP: One mechanism by which EEP reduces Glu-induced neurotoxicity might be by interfering with $Ca^{2+}$ influx through Glu receptor-gated membrane channels. To investigate the possibility that EEP attenuates Glu-stimulated increases in $[Ca]_i$ in vitro by an action at the NMDA or kainate types of Glu receptor, neurons were loaded with the $Ca^{2+}$ -sensitive dye indo-1 and exposed to Glu (0.5μM) following a 2 min. pretreatment with either TRH or EEP. A submicromolar concentration of Glu was chosen to avoid failing to detect an

effect of the tripeptides due to the overwhelming and sustained increases in $[Ca]_i$ seen at higher Glu concentrations. As indicated in Table 1, 500 nM Glu typically caused an immediate increase in $[Ca]_i$ of 282 ± 16 nM, but this gradually dropped during the ten min course of the experiments to a lower level approximately 67 ± 5nM above baseline. When neurons were pre-incubated with 500μM TRH, the initial Glu induced rise $[Ca]_i$ was significantly reduced by over 50% to 122 ± 17nM. In contrast, neurons pre-incubated with the same concentration of EEP had a much less dramatic effect reducing the initial Glu-induced rise in $[Ca]_i$ by just under 25% to 213 ± 18nM. Interestingly, doubling the concentration of EEP to 1000μM resulted in very nearly the same level of reduction as was seen with 500 μM TRH. Under these conditions, the initial Glu induced elevation of $[Ca]_i$ was reduced by more than 60% to 109 ± 15 nM. There were no significant changes in the post-peak levels of $[Ca]_i$ under any of the conditions.

4. Conclusion: We have verified that EEP is neuroprotective under the above identified conditions in cultures derived from both forebrain and spinal cord, and we have shown that EEP is relatively more effective than TRH in this model.

5. I further declare that all statements made herein or my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

Dated: 13 FEB 2003 Michael L. Koenig

Michael L. Koenig, Ph.D

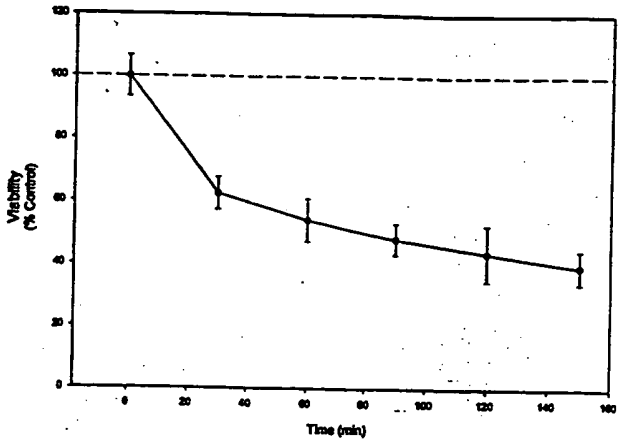


Fig. 1. Glutamate-induced neurotoxicity as a function of time. Neurons were treated with Glu (100 $\mu$M) for 30, 60, 90, 120, and 150 min. Viability is expressed as % Control (0 min), and data are presented as means ± SD. A 90 min exposure results in the death of approximately half of the neurons (48.1 ± 5.0% of mean control value).

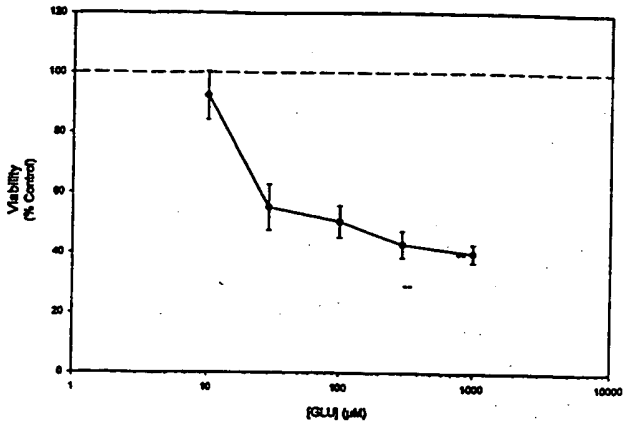


Fig. 2. Concentration dependence of Glu-induced neurotoxicity. Neurons were treated with 10, 30, 100, 300, and 1000 $\mu$M Glu for 90 min. Viability is expressed as % Control (no Glu), and data are presented as means ± SD. Exposure to 100 $\mu$M Glu for 90 min results in the death of approximately half of the neurons (50.5 ± 5.3% of mean control value).

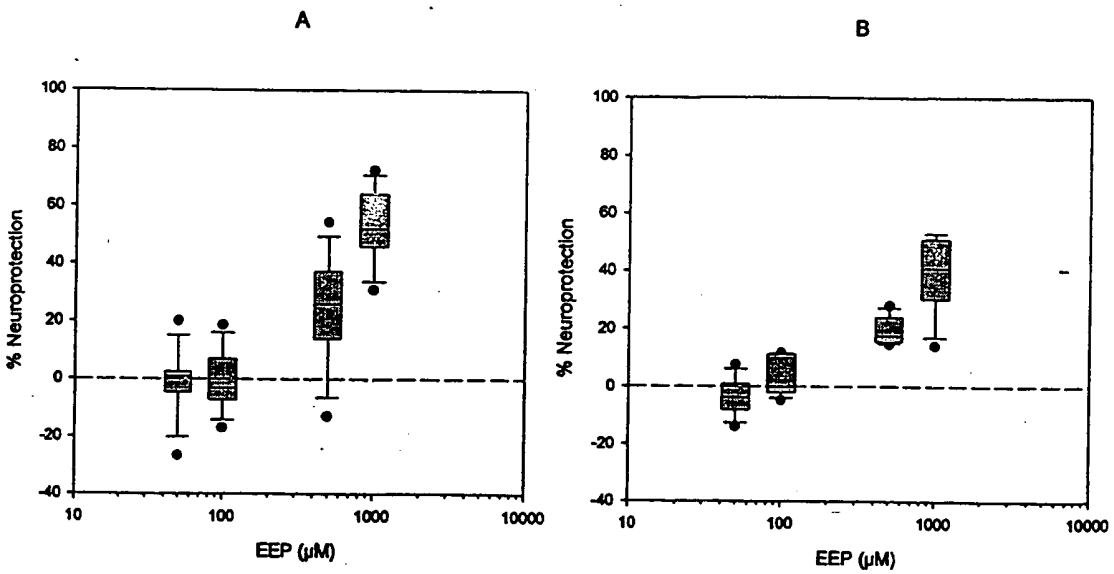


Fig. 3. Neuroprotection by EEP in neurons derived from forebrain (panel A) and spinal cord (panel B). Neurons were exposed to 100 $\mu$M Glu and the indicated concentrations of EEP for 90 min. 24 h later neuronal viability was assessed by MTT assay. Percent neuroprotection is presented on the ordinate; concentrations of EEP are depicted on the abscissa. Data are displayed as box plots (box includes median and middle two quartiles; error bars extend to 10th and 90th percentiles).

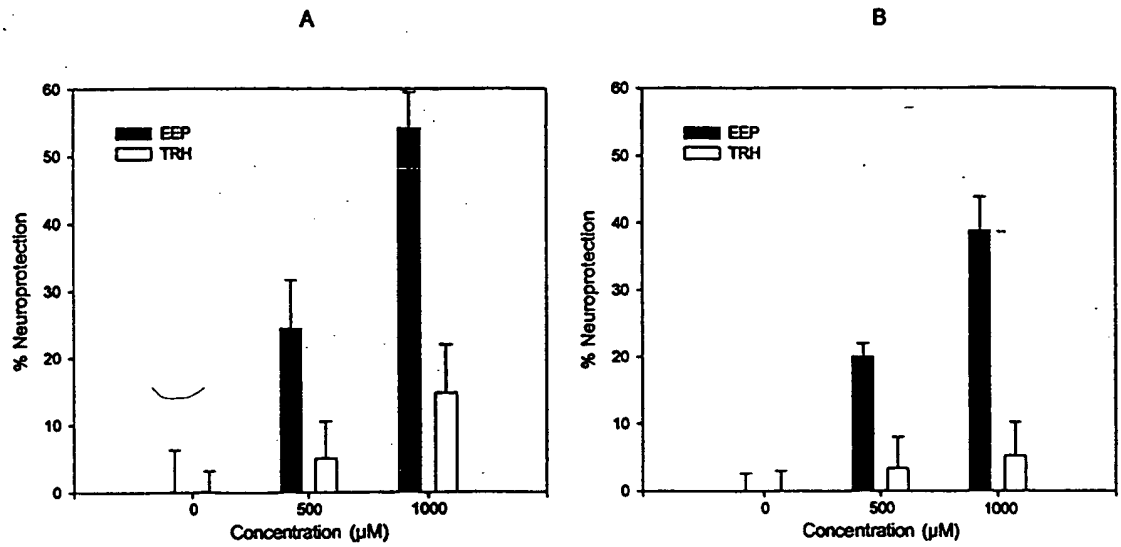


Fig. 4. Comparison of neuroprotective efficacies of EEP and TRH in neurons derived from forebrain (A) and spinal cord (B). Neurons were exposed to 100 μM Glu and the indicated concentrations of EEP (filled bars) or TRH (open bars). 24 h later the percent neuroprotection was assessed by MTT assay. Bars represent the means ± sem at each concentration.

Table 1
Reduction of Glu-stimulated increases in [Ca] by TRH and EEP

| Treatment | $\Delta[Ca]_i$ (nM) | | n |
|---|---|---|---|
| | (Peak)-(Basal) | (Post-peak)-(Basal) | |
| Glu (500 nM) | 282 ± 16 | 67 ± 5 | 204 |
| TRH (500 μM) + Glu | 122 ± 17*** | 115 ± 13$^{ns}$ | 44 |
| EEP (500 μM) + Glu | 213 ± 18*** | 96 ± 11$^{ns}$ | 146 |
| EEP (1000 μM) + Glu | 109 ± 15*** | 64 ± 8$^{ns}$ | 71 |

Neurons were treated with Glu (500 nM) following pre-incubation with Locke's bathing medium (control), TRH, or EEP. The results depicted in this table reflect the differences in $[Ca]_i$ relative to basal levels. The data are presented as means ± sem. Differences in the means relative to the values for Glu were tested by one-way ANOVA and the Bonferroni multiple comparisons test.

*** Indicates $p < .001$.